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The rediscovery of smallpox

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Abstract

Smallpox is an infectious disease that is unique to humans, caused by a poxvirus. It is one of the most lethal of diseases; the virus variant *Variola major* has a mortality rate of 30%. People surviving this disease have life-long consequences, but also assured immunity. Historically, smallpox was recognized early in human populations. This led to prevention attempts—variolation, quarantine, and the isolation of infected subjects—until Jenner's discovery of the first steps of vaccination in the 18th century. After vaccination campaigns throughout the 19th and 20th centuries, the WHO declared the eradication of smallpox in 1980. With the development of microscopy techniques, the structural characterization of the virus began in the early 20th century. In 1990, the genomes of different smallpox viruses were determined; viruses could be classified in order to investigate their origin, diffusion, and evolution. To study the evolution and possible re-emergence of this viral pathogen, however, researchers can only use viral genomes collected during the 20th century. Cases of smallpox in ancient periods are sometimes well documented, so palaeomicrobiology and, more precisely, the study of ancient smallpox viral strains could be an exceptional opportunity. The analysis of poxvirus fragmented genomes could give new insights into the genetic evolution of the poxvirus. Recently, small fragments of the poxvirus genome were detected. With the genetic information obtained, a new phylogeny of smallpox virus was described. The interest in conducting studies on ancient strains is discussed, in order to explore the natural history of this disease.

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Introduction

Smallpox (variola) is an acute infectious disease of viral origin (genus *Orthopoxvirus*) that has caused devastating epidemics. During the 20th century, it was responsible for 300–500 million deaths, and in the last millennium it is estimated to have been responsible for 10% of deaths worldwide. Thus, it is highly probable that this disease has strongly selected human populations, but there is no current evidence to validate this hypothesis. Following a global pandemic in the 18th century, vaccination against smallpox began in the early 19th century, and the last case was reported in Somalia in 1977. In 1980, the WHO declared that smallpox had been eradicated. It is not clear what role population selection and the general improvement in hygiene conditions played in this eradication process, but the extensive vaccination programme conducted by the WHO certainly accelerated the process (www.who.int/csr/disease/smallpox/en/). In the modern era, although populations worldwide have been affected by smallpox, it is the isolated non-occidental populations that have been affected the most; some New World and Siberian populations have been decimated by the virus.

Today, the study of smallpox has various challenges. (i) Social and medical challenges: many populations living in remote areas, where smallpox has become endemic only recently, will migrate to live in urban or suburban environments. Thus, minor cases of the disease could result in epidemics through the gradual reduction in the number of vaccinated people, and the major environmental changes that affect them. Moreover, humans constitute the only known

reservoir of the virus, but the emergence of an animal reservoir or a very closely related animal virus that could adapt to humans is not excluded. Note that current animal poxvirus strains can infect humans, but that virulence differs between strains [1]. Governments must therefore control live strains of the poxvirus (see below), and remain informed about the hazards of the virus, its mutation potential during epidemics (which can speed up the epidemic and/or make it last longer), and the potential for competition between different strains. It is also necessary to identify these strains, and to distinguish between the natural re-emergence of the disease and its genetic manipulation in the laboratory. This is also of importance because smallpox could be developed for acts of bioterrorism. (ii) It is also a biological challenge, owing to co-evolution between humans and the environment. What genes were selected by smallpox epidemics during human evolution? How has the virus evolved? In human population histories, what were the relationships between the major human-killers (plague and tuberculosis) and smallpox?

Recently, we demonstrated a case of smallpox through molecular analysis; an autochthonous subject who died in Yakutia, Eastern Siberia (Fig. 1), between 1628 and 1640, during a significant tuberculosis epidemic [2]. On the basis of small fragments of degraded DNA, we proposed a new phylogeny of the virus. This research gave new perspectives on the genetic study of smallpox; ancient smallpox samples are now considered to be safe to work on, owing to the degraded nature of their DNA. There is no doubt that the discovery of ancient smallpox cases and the sequencing of ancient strains will give further insights into the natural history of this disease.

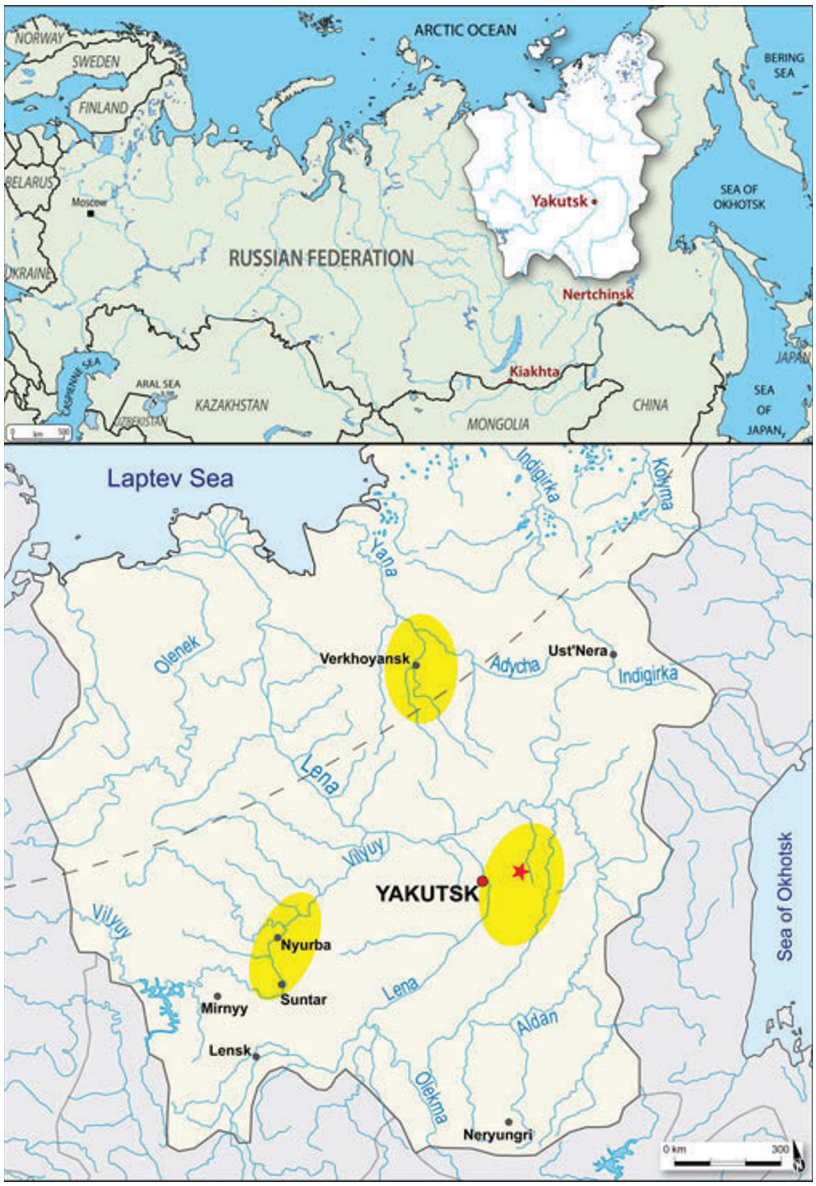

FIG. 1. Location of Yakutia in Siberia. Map of the three initial regions occupied by ancient Yakut populations excavated during MAFSO archaeological campaigns. The star shows the location of the shamanic tree grave, © Patrice Gérard CNRS.

Clinical Symptoms and Epidemiology

Humans are the only known hosts or reservoirs of smallpox. Smallpox is one of the most feared diseases in the world, as its mortality rate ranges from 1% for cases of *Variola minor*, to >97% for haemorrhagic smallpox cases in unvaccinated subjects. The incubation period of 8–14 days is followed by a virus-like invasion phase (high fever, shivering, and arthralgia), and then by a state phase with a maculopapular rash, which produces blisters on the third day and infected pustules on the seventh day. If the subject survives, there is a drying phase on the 10–12th days, when the scabs fall off to leave permanent scars. The patient will then be immunized, but the duration of this immunization period is still debated [3]. With the haemorrhagic form, the subject dies after the first 24 h of the invasion phase, following bleeding from the skin and/or mucous membranes. During the 19th century, less virulent smallpox epidemics were recorded, with a death rate of 1% in South Africa and Central America, where the less virulent smallpox is known as alastrim [4]. The mode of disease transmission is usually direct contact: the virus is transmitted primarily by the exhalation of patients, and then by contact with skin lesions. The virus is very stable, and can survive for years in the scabs [5]. In natural conditions, however, the virus remains pathogenic for a couple of weeks.

The earliest and most incontestable descriptions of smallpox appeared in the fourth century in China, and then later in India (seventh century) and in Southwest Asia and the Mediterranean (tenth century) [4]. It was prevalent in southern Europe during the 13th century, when it started to spread throughout Europe, causing millions of deaths over the centuries [6,7]. In the 18th century, smallpox was endemic in Europe, and significant peaks in the number of deaths were observed—14 000 in 1716, 20 000 in 1723 and 14 000 in 1796 in Paris [9]—killing 10% of newborns and one in three adult patients. It decimated Amerindian populations during the conquest of the New World from the beginning of the 16th century [4], and it was the succession of smallpox epidemics and other infectious diseases imported from the Old World that destroyed what remained of the Inca culture [8].

Owing to its distinctive clinical symptoms, smallpox has been known as a disease 'entity' for many centuries. Campaigns against smallpox began in the absence of current scientific knowledge, while the causes and mechanisms of the disease were unknown. There were attempts to develop various methods to protect against smallpox. The earliest methods, reported in the tenth century, were insufflation in China and cutaneous inoculation in India [4]. Variolation, to avoid serious smallpox, consists of an inoculation with 'benign smallpox' from the pustules of a patient. The origin of this technique is disputed, but it is known to have been practised in China in the 16th century. It was imported into the West in the early 18th century by Lady Wortley Montagu, the wife of the Ambassador of Great Britain to Turkey [10], but it is likely that different techniques had existed previously in the European countryside. In Scotland, farmers attached wool soaked in smallpox pus to their wrists, and in some Italian mountain areas children were made to wear similarly soaked shirts [11,12]. The results were, however, very random, and many subjects still died, despite these attempts at variolation. The practice was introduced into France in the second half of the 17th century, where it was extensively studied and fiercely debated (owing to the dangers of inoculation), with written submissions to the Academy of Sciences [4]. Before 1760, European doctors replaced the needle (used in Turkey) with the lancet, which allowed doctors to make a deeper cut [13], but these incisions were either ineffective or catastrophic. After 1760, Sutton proposed a method involving a more superficial incision, increasing the reliability of the inoculation and hence its popularity [12].

From 1769, at least six people in England and Germany successfully tested the possibility of using the cowpox vaccine against smallpox in humans [4]. At the end of the 18th century, Edward Jenner, a country doctor in England with many farmer patients, noted that contact with livestock, particularly cows, exposed farmers to a disease that was transmissible to humans —cowpox or vaccinia, the smallpox of cows. From the observation that milkmaids who had contracted cowpox did not usually contract smallpox, Jenner postulated that the pus present in the cowpox vesicles protected people against smallpox. Today, we know that cowpox vaccine causes a benign disease in humans, in comparison with smallpox, but at the time it seemed that a convergence of ideas led to replacing the pus of smallpox with that of cowpox.

In 1796, Jenner developed the first stages of the vaccination process: he inoculated a child with the pus from the hand of a farm worker who was infected with cowpox (via contact with the infected cow's udders), causing a localized infection at the inoculation site. Three months later, he inoculated the child with smallpox; the child showed no signs of infection, and so was immunized against the virus [4]. Over a period of 20 years, Jenner conducted systematic experiments that led to the establishment of the vaccination method, which replaced inoculation. It should be noted, however, that, at the same time as variolation and vaccination, procedures were being used in Europe, the quarantine and isolation of affected individuals, which also reduced the spread of the disease [14,15].

In the early 19th century, vaccination against smallpox spread rapidly throughout the occidental world [16], and was

largely responsible for the observed change in world demography. World demography before vaccination is termed pre-Jenner; in the course of several generations, for 1000 births, one-third of subjects died between birth and the age of 1 year, and one-third between 1 year and 18 years of age. World demography after vaccination is termed post-Jenner; an increase in the number of surviving subjects between birth and 18 years of age is observed. If birth rates remain constant, there is a population explosion, and this is what occurred in Europe in the 19th century because of vaccination. The world population increased to approximately 1 billion in 1800, and to >1.5 billion in 1900 [17]. The first vaccination campaigns, carried out in the first half of the 20th century, and the WHO screening programme conducted from 1967 onwards were highly successful, and smallpox was declared to be eradicated in 1980 (the last reported case was in 1977 [4].

Initial Isolation and Characterization of the Smallpox Viruses

The disease was recognized and measures for its prevention were initiated early in human history (16th century in China), but investigations into the virus itself did not begin until the late 19th century, with the establishment of the new science of virology. Virus particles of smallpox, cowpox and vaccinia were first identified under the microscope, and then by electron microscopy (before any other viruses had been visualized), and their chemical compositions were analysed before those of animal viruses [4]. Most studies and knowledge on orthopoxviruses were based on the vaccinia virus.

The first reports of studies on the viral elements of vaccinia using microscopy were in 1886 by Buist [18] and Calmette-Guérin in 1901 [19], who observed many elementary particles in the stained smears and vaccine lymph of rabbits. Prowazek in 1905 and Paschen in 1906 [4] also showed these 'elementary bodies' by using different microscopy methods. It was, however, the work of Ledingham in 1931 [20] that demonstrated definitively that the elementary bodies were indeed the infectious entities in the lymph. Thereafter, analysis of the pox virion structure was dependent on the power of electron microscopy. Between 1948 and 1970, thin sections of infected cells were used to characterize the membrane structures, core and components of the vaccinia virus [4].

The presence of DNA in the virus, but not RNA, was demonstrated in 1940 [21,22]. In 1962, the double-stranded nature of the virus DNA and its guanine and cytosine composition was demonstrated [23]. The first genomic sequences of the smallpox virus were published in the early 1990s, with the complete DNA sequence of the vaccinia virus [24,25]. Thereafter, a significant effort was made to sequence most of the isolates that had been collected during the WHO Intensified Smallpox Eradication Programme (1967–1980). Currently, approximately 50 genomes of human poxviruses, as well as those of dozens of animal poxviruses, are available on the web (http://www.poxvirus.org). Thus, new insights into the origin, distribution and evolution of different human clades have been obtained [26,27].

The Virus and its Evolution

Viruses of the genus *Orthopoxvirus* are widespread in vertebrates, and most of them are species-specific [28,29]. The best-studied members of the *Orthopoxvirus* are the poxviruses (*Poxviridae*), DNA viruses that replicate in the cell cytoplasm [30]. These include smallpox viruses (*Variola major* and *V. minor*), the primate viruses (highly pathogenic to humans), and the bovine poxvirus (used as a vaccine against the three aforementioned viruses). The genome of an orthopoxvirus is a linear double-stranded DNA molecule containing *c.* 200 genes. The central region of the genome has highly conserved sequences, and contains the majority of genes involved in transcription, mRNA biosynthesis, DNA repair and replication, and the modification of viral structural proteins [29,30]. The terminal regions are variable, and determine the specific properties of the species, the strain of the virus, and its immunomodulatory protein genes [31–33]. The virulence of smallpox is related to a set of genes encoding proteins that efficiently modulate (as compared with other viruses) the multiple defence mechanisms of the host organisms [34].

Following the eradication of smallpox in 1980, two conservatories holding the live viral strains were created: in the CDC in Atlanta, USA, and VECTOR in Novosibirsk, the Russian Federation. Although the purpose of these conservatories is regularly discussed [35], their goal is to store live strains in order to establish medical and vaccination strategies in the event of the re-emergence or bioterrorism. Currently, they store live smallpox strains that were collected between 1944 and 1977 [26,36]. Their genomes have been completely sequenced and are open access (NCBI GenBank). These strains follow the global phylogeographical distribution; the first cluster groups *V. major* strains from Asia, India, and Africa, excluding the West, and the second cluster groups the two subclusters of West Africa and South America.

Several studies on the strains are being conducted by the WHO and authorized laboratories: tests for the diagnosis of smallpox in the laboratory, smallpox genomic studies, the establishment of animal models and of pathogenesis, and the development of antivirals for the treatment of smallpox [37].

Independent advisory experts assess all smallpox research programmes [38]. With technological advances in high-throughput sequencing and the generation of whole genomes for modern strains, the origin of and variability in virulence factors for these poxviruses are being well studied with phylogenetic methods [26,36]. The evolution of different smallpox viruses is being studied by the use of data on sequence diversity, genome structure, and variation in coding regions [27]. The evolution and phylogeny of poxviruses specific to vertebrates are also being studied, to obtain a better understanding of the dispersion and date of the origin of these strains [39,40].

The published phylogenetic tree found a root/most recent common ancestor (MRCA) for the sequences of AD 120 (maximum) (Fig. 2; mean estimate of AD 928 for the the MRCA of all smallpox sequences; 5–95% confidence range of AD 120 to AD 1714; median of AD 1585). This date is consistent with that reported in early documents on smallpox (before AD 1000 [4]), but it is certainly very recent, considering when the disease first appeared (between 1500 BC and 1100 BC in China and India [4]). Indeed, apart from the traditional descriptions of the disease, interpretations of certain texts and/or archaeological discoveries suggest a much earlier existence of the disease, especially in its cutaneous form. Typical rashes have been identified in Egyptian mummies dating from 1100 BC to 1580 BC [4,41,42], and the disease could have struck China in 1122 BC and India in 1500 BC [4,42]. The divergence between current phylogenies and archaeological evidence is related to the fact that current strains represent only a small part of smallpox genetic variability in the 20th century. This has

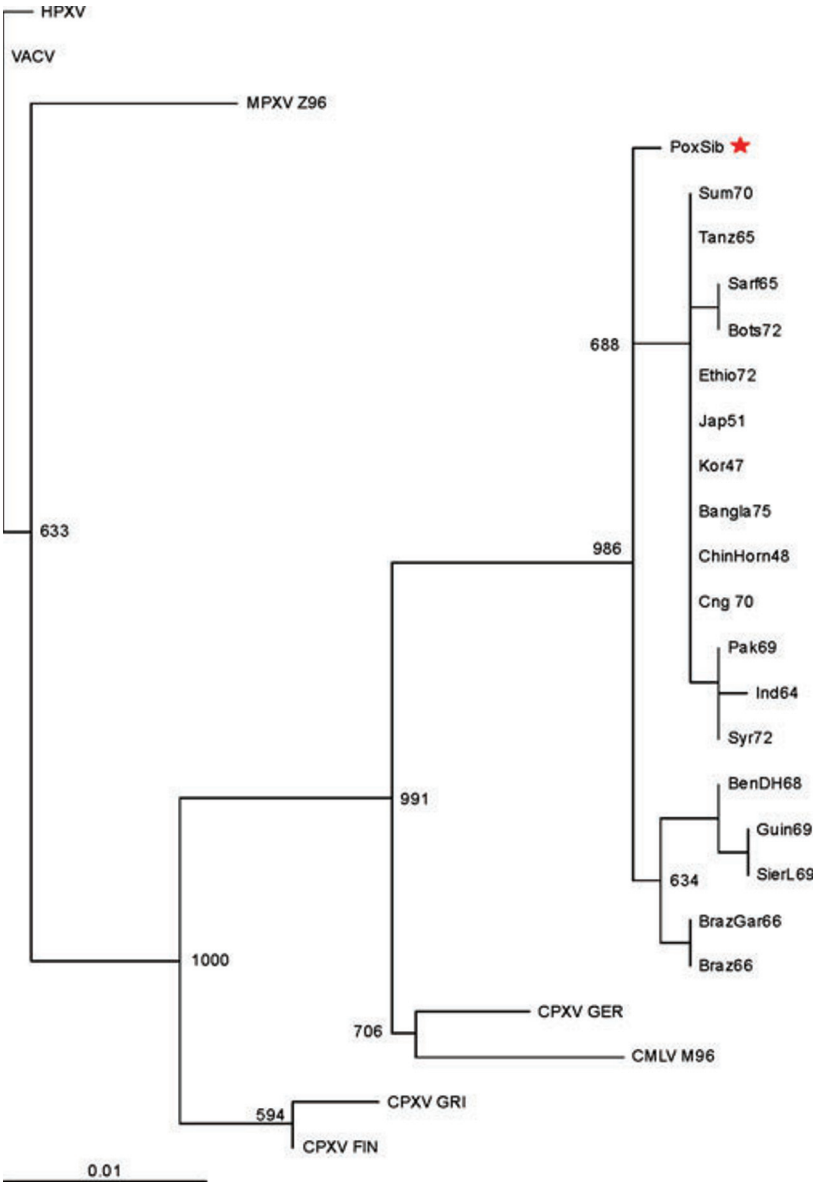

FIG. 2. Phylogenetic analysis of concatenated sequences of an ancient Siberian smallpox virus (PoxSib). These three gene segments showed that the 300-year-old virus did not belong to the cluster of known sequenced strains of the 20th century (1946–1977). Derived from Fig. 1b in [2]. Reprinted with permission.

several implications: (i) as more strains are discovered, variability increases, so it is probable that the MRCA is older than present estimates suggest; and (ii) a large number of strains have disappeared during human history, or they are not detected today, because they are no longer virulent. Strains affecting European and American human populations between the 13th and 18th centuries have been eradicated since the 19th century, following the first vaccination campaigns [7].

Palaeogenetic Studies

Palaeomicrobiology is a field of research based on the detection of infectious diseases in the past [43]. Fragments of bacterial or viral DNA are searched for in possibly infected tissues, or in the teeth of subjects, who died while they had bacteraemia or viraemia, taking into account the specific characteristics of the ancient DNA sample (Fig. 3). Regarding smallpox, our work in Central Yakutia (Eastern Siberia) in 2004 led to the discovery of an elite burial (wealthy and organized) dating from between 1730 and 1740, with five remarkably well-preserved subjects (Fig. 4). The anatomical study revealed no trauma, and the burial of subjects at the same time during winter suggested that they had died during a disease epidemic [44]. The search for pathogens was initially focused on bacterial pathogens, but as none was found, a viral origin was hypothesized. This was reinforced by the presence of iron in the lung alveoli of the subject, suggesting death resulting from a haemorrhage. Smallpox was not the first virus to be tested for, because none of the four subjects whose skin could be examined showed any traces of blisters or pustules. Only in the best-preserved subject were we able to identify

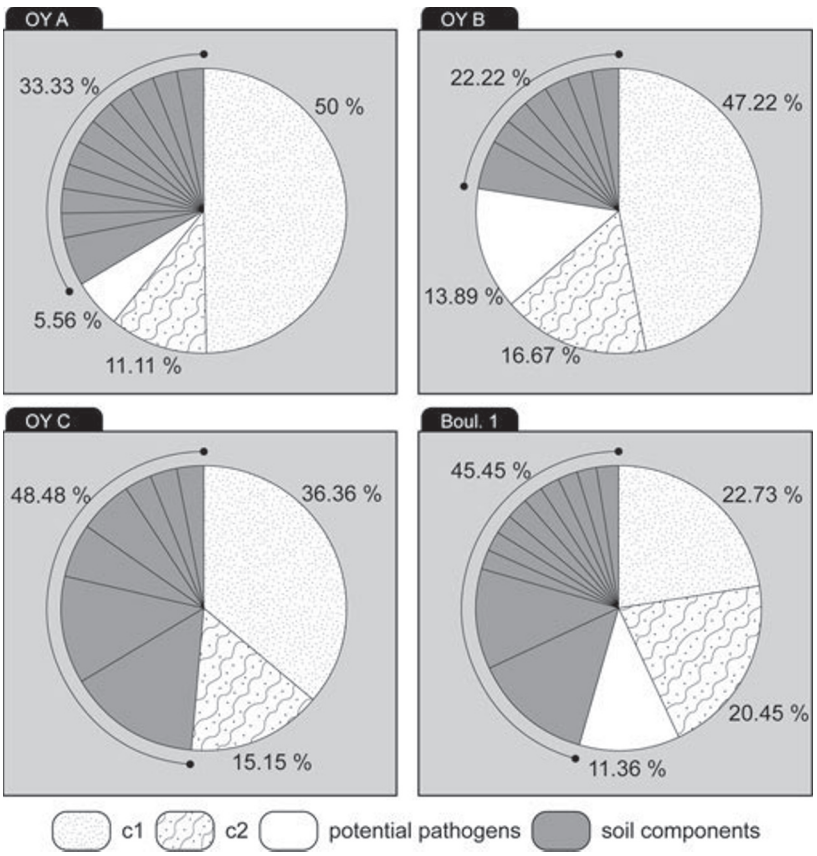

FIG. 3. Composition of environmental, undetermined and potential pathogenic 16S rDNA sequences in ancient skeletons and frozen bodies. The majority of the bacterial pool comprised environmental bacteria originating from the soil, vegetation or permafrost characteristic of the Arctic. Reproduced from Fig. 2 in [45]. Reprinted with permission. Palaeogenetic studies conducted over >10 years in our laboratory have clearly demonstrated the persistence of fragmented DNA molecules [45,47]. It is important to note that such archaeological samples consist of many DNA molecules, originating not only from the subject (human nuclear and mitochondrial DNA) and the pathogen potentially responsible for the death of the subject (bacterial or viral DNA), but also environmental bacteria from this complex environment: soil, water, and plants. Metagenomic or bacterial genome analyses show that the majority of bacteria in this kind of sample are environmental [45].

FIG. 4. Evocation of the shamanic tree grave, © Nicolas Senegas; watercolour of different masks and capes of studied woman, © Christiane Petit-Hochstrassesr. The tomb is large and well organized, which shows that the population and the related subjects had not all been eradicated. It is interesting to note that the golden age in Yakutia ends after 1728–1740 [44,46]. Originally, we discussed a major economic change [44]; it would be interesting to determine how a haemorrhagic smallpox epidemic could have participated in this decline.

three DNA fragments of the ancient human poxvirus by PCR [2]. To assess the persistence of large DNA fragments, we conducted long-range PCRs on the E9 l gene (coding for polymerase), according to the method in [39]. The amplifications expected were approximately 1000 bp and 2043 bp: no positive results were obtained, suggesting a fragmented viral genome [2]. The absence of pustules, the presence of a pulmonary haemorrhage and the detection of the virus could indicate that the subject had haemorrhagic smallpox. It would be interesting to look for the presence of smallpox in the other subjects in the grave (Fig. 4). The first phylogenetic study conducted on these fragments showed, as expected (see above), that this ancient strain is distinct from the modern strains described previously (Fig. 2).

This study was the first of its kind—it opens up interesting avenues for phylogenic and natural history approaches to studying smallpox. The virus is considered to be resistant to environmental conditions, but the subject was buried in Central Yakutia, where temperatures can drop below 50°C in winter (see conservation of burials and bodies in Fig. 5), and a burial lasting two and a half centuries has destroyed the virus, fragmenting and degrading its DNA. It is hoped that research on archaeological samples will be facilitated in the future. According to current legislation and risk assessments, the theoretical level of containment required in a laboratory, owing to the classification of a parental strain, does not necessarily need to be implemented. Smallpox and archaeological samples that are significantly degraded are not pathogenic. Thus, we can assume that a biosafety level of 2 would be

FIG. 5. The frozen graves of Yakutia: an exceptional conservation. From left to right: Shamanic tree (2004), Kouranakh (2011), Ordiogone 2 (2007), © Patrice Gérard CNRS; Eletcheï 1 (2009), © Nicolas Senegas. The main ethnic group from this region, the Yakuts, used inhumation as a common funerary practice from when they discovered the region in the Middle Ages [44]. This is unique to Yakuts in Siberia and the Arctic, and provides a unique opportunity for continuous temporal sampling from the Middle Ages onwards.

required in a laboratory, corresponding to the minimum level required by the regulations for handling of samples of human origin (e.g. teeth). The scientific community could then use the 'smallpox model' to assess the co-evolution of virulence and resistance genes (if they exist in humans) in subjects buried before, during and after an epidemic. The future of smallpox natural history remains to be written; it will not be dangerous, so the interest in keeping live strains of the virus is brought into question again.

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Transparency Declaration

The authors declare no conflicts of interest.

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